

## CHARACTERIZATION OF ANTIHYPERTENSIVE PEPTIDES FROM *PLEUROTUS CYSTIDIOSUS* O.K. MILLER (ABALONE MUSHROOM)

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### ABSTRACT

The antihypertensive activities of the crude water extracts from nine fruiting bodies of edible mushrooms were screened. The ACE inhibitory activities of the water extracts at a concentration of 10 mg/ml were in the range of 71.9% to 95.5%. Following this, proteins were precipitated from the crude water extracts using ammonium sulphate precipitation method from 10% to 100% salt saturation (S10-S100). The crude proteins were grouped according to the protein bands resolved by SDS PAGE. The ACE inhibitory activity of the protein fractions were tested at a concentration of 10 µg/ml protein. Fraction 1 of *P. cystidiosus* (S10-S20) had the highest activity where 96.2% of ACE was inhibited. This was followed by fraction 5 of *P. cystidiosus* (S90) where 86.7% of ACE was inhibited. Thus, fraction 1 and fraction 5 of *P. cystidiosus* were selected for further purification by reversed phase high performance liquid chromatography (RPHPLC). The proteins were eluted at an increasing gradient of acetonitrile in 0.05% TFA from 0 to 50% in 10 min. The protein fractions were grouped into sub-fractions. The ACE inhibitory activity of the RPHPLC fractions was tested at 1 µg/ml. Fraction F1a and F5b from fraction 1 and fraction 5 respectively showed the highest ACE inhibitory activity. These fractions were further characterized using SELDI-TOF-MS analysis. The result obtained showed the fractions contained several low molecular weight proteins where the most abundant protein had a molecular weight of 8300 Da.

**Keywords:** Low molecular weight proteins, ACE inhibitory peptide, SELDI

### INTRODUCTION

Hypertension or high blood pressure has been on the rise in Malaysia over the past 10 years [1]. Hypertension is one of the risk factors for cardiovascular diseases, such as stroke, heart failure, cardiac arrhythmia and arteriosclerosis [2]. The most common blood pressure control has been associated with the renin-angiotensin system. Renin (protease synthesized by kidney) converts angiotensinogen from the liver to angiotensin I, which is a biologically inactive decapeptide. Later, angiotensin I is converted to an active octapeptide vasoconstrictor, angiotensin II, by the action of angiotensin I-converting enzyme (ACE). This reaction will cause the contraction of blood vessels and thereby raising blood pressure. Thus, blocking the production of angiotensin II with ACE inhibitors will prevent constriction of blood vessels, lowers blood pressure and lessens the energy of the heart to expand when beating [3]. Besides blood pressure lowering effect, ACE inhibitors have also shown improvement in the treatment of several clinical disorders such as memory dysfunction, cerebral blood flow and cerebroprotection, stress, depression, alcohol consumption, seizure, Alzheimer's and Parkinson's diseases and diabetes [4]. Various ACE inhibitors commercially available in the market, such as captopril may effectively help to regulate the blood pressure in hypertensive patients. However, these pharmaceutical products may have side effects such as cough, taste disturbances, skin rashes and allergic reactions.

Previous study has reported the occurrence of angioedema caused by the consumption of ACE inhibitors [5]. Thus, the interest to find ACE inhibitors from natural sources such as food has increased. Many ACE inhibitory peptides have been discovered, among which some are derived from food-proteins such as milk, buckwheat, potato and tuna [6-9].

Mushrooms have received increasing attention in recent years because of their nutrition with health-stimulating properties and medicinal effects. Beside nutritional value, the unique colour, taste and aroma of the mushrooms which can sometimes stimulate one's appetite are also the reasons that attract their consumption by humans [10]. It is not easy to separate edible and medicinal mushrooms because many of the common edible species are beneficial in the prevention and treatment of various human diseases and several medically related mushrooms are also eaten [11]. The most reported medicinal mushroom with blood pressure lowering effect is *Ganoderma lucidum* [12, 13]. Previous studies have also reported on the antihypertensive effect of some edible mushrooms. The low concentration of sodium and the presence of a high amount of potassium in mushrooms have suggested its utilisation as an antihypertensive diet [11]. Potassium has been proven to have blood pressure lowering effect [14]. *Pleurotus* species such as *P. cornucopiae*, *P. nebrodensis* and *P. sajor-caju* have been reported to possess hypotensive activity [15-17]. *Lentinula edodes* and *Flammulina velutipes* have also been reported to exert antihypertensive activity [18, 19]. Previous studies have showed successful purification of ACE inhibitors from fruiting bodies of edible mushroom, *Tricholoma giganteum* and *Grifola frondosa* [20, 21]. Excess production of reactive oxygen species (ROS) will contribute to hypertension. Antioxidant administration may help to prevent and treat hypertension [22]. *Pleurotus cystidiosus* has strong antioxidant effect [23]. Therefore, it may be a good source of an antihypertensive drug. Thus, the objective of the current study is to extract and characterize the ACE inhibitory peptide from the fruiting body of *P. cystidiosus*.

## MATERIALS AND METHODS

**Materials and Chemicals.** Fresh mushroom fruiting bodies: *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (golden needle), and *Lentinula edodes* (shiitake) were purchased from the local hypermarket, Jaya Jusco while *Hericium erinaceus* (monkey's head mushroom), *Pleurotus citrinopileatus* (yellow oyster mushroom), *P. cystidiosus* (abalone mushroom), *P. flabellatus* (pink oyster mushroom), *P. florida* (white oyster mushroom) and *P. sajor-caju* (grey oyster mushroom) were purchased from the local mushroom farm, Ganofarm Sdn Bhd. Captopril, a commercial antihypertensive drug and the  $\alpha$ -cyano-4-hydroxy-cinnamic acid (CHCA) used for SELDI-TOF-MS was purchased from Sigma-Aldrich. Acetonitrile used for HPLC and the other chemicals such as ammonium sulphate, trifluoroacetic acid (TFA) and methanol were purchased from Merck.

**Partial Purification of ACE Inhibitor.** The fruiting bodies were homogenized with distilled water at a ratio of 1:2 (w/v). The mixture was filtered and centrifuged to remove the unwanted debris. Proteins were precipitated out from the water extracts using ammonium sulphate precipitation method. The concentration of the ammonium sulphate was increased stepwise from 10% to 100% salt saturation and the precipitated protein was recovered at each step by centrifuging at 10000 rpm for 15 min. Then, the crude protein was dialyzed to remove the salt from the sample. They were lyophilized and stored at -20°C for further analysis.

**Protein Estimation.** Protein content was estimated using Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) according to the protocols provided by the manufacturer. Absorbance of the samples was measured with Sunrise™ ELISA microplate reader (Tecan) at

562 nm. Protein content was determined by comparing the absorbance value of the samples with the standard curve of bovine serum albumin (BSA).

**SDS Polyacrylamide Gel Electrophoresis (SDS PAGE).** SDS PAGE was carried out in a vertical slab gel apparatus according to the modified method of Laemmli (1970) [24]. Sample buffer and crude protein were mixed at a ratio of 1:3 (v/v) and boiled for 5 minutes. The mixture and broad range SDS PAGE standard markers (Bio-Rad) were then loaded into the well. Electrophoresis was conducted at a constant current of 60V for stacking gel (4% polyacrylamide) and followed by 100V for separating gel (16% polyacrylamide). After electrophoresis, the gel was fixed with fixing solution which consists of 40% (v/v) methanol and 10% (v/v) acetic acid for 30 min. The protein bands were then stained by silver staining.

**Assay of the ACE Inhibitory Activity.** The protein fractions were tested using angiotensin converting enzyme inhibitory assay kit (ACE kit-WST, Dojindo Laboratories). The crude mushroom extracts and the protein fractions were tested at a concentration of 10 mg/ml and 10 µg/ml protein, respectively. The assay was carried out according to the protocol provided by the manufacturer. Absorbance of the samples was measured with Sunrise<sup>TM</sup> ELISA microplate reader (Tecan) at 450 nm. The ACE inhibitory activity of the samples tested was calculated using the formula given in the protocol.

**Purification of ACE Inhibitor from *Pleurotus cystidiosus*.** Purification of the protein fractions with active ACE inhibitory activity was carried out using HPLC system (Shimadzu, SPD-M10A UP). The column used in the current study was Chromolith® SemiPrep RP-18 column (100-10 mm, Merck KGaA, Darmstadt, Germany). First, the crude proteins were filtered through 4 mm X 0.45 µm syringe filters (Whatman) before injected into the column. The crude proteins were eluted with an increasing gradient of acetonitrile containing 0.05% TFA, from 0% to 50% in 10 min at a flow rate of 3.5 ml/min. The UV absorbance of the eluent was monitored at 220 nm. All the protein fractions were sub-fractioned according to the peaks obtained. They were concentrated by lyophilisation and the antihypertensive activity of the purified protein fractions was determined at a concentration of 1 µg/ml protein.

**Surface-Enhanced-Laser-Desorption-Ionization-Time-of-Flight-Mass-Spectrometry (SELDI-TOF-MS) Analysis.** Protein fractions of F1a and F5b of *P. cystidiosus* were analysed using hydrophobic H50 ProteinChip® arrays (Bio-Rad Laboratories, Inc). The arrays were first equilibrated with binding buffer, which consist of 0.5% TFA in 50% acetonitrile (v/v). Then, 5 µl of samples containing 0.5 µg of protein were spotted on the arrays. After the arrays were air-dried, 2 µl of CHCA in 0.5% TFA in 50% acetonitrile (v/v) was added onto the arrays. The arrays were left to air-dry. The analyses were carried out with the ProteinChip SELDI system (Bio-Rad Laboratories Inc., PSC 4000). Data collection was carried out in positive ion mode using the following acquisition settings: mass range of 0 to 20 kDa, focus mass of 10 kDa. Laser energy used for the shot sequences were based on the following settings: warming shots 1000 nJ and data shots 900 nJ. Spectra were calibrated using an external calibration against a mixture of standards consisting of somatostatin (1637.9 Da), arg-insulin (5969.0 Da) and cytochrome c (12263.3 Da).

## RESULTS AND DISCUSSIONS

**ACE Inhibitory Activities of Water Extracts from Fruiting Bodies of Edible Mushrooms.** Water extracts from the fruiting bodies of the edible mushrooms were tested for their ACE

inhibitory activity at a concentration of 10 mg/ml. As shown in Table 1, the nine edible mushrooms tested showed high ACE inhibitory activity where 71.9 to 95.5% of ACE was inhibited. Among the mushrooms tested, *F. velutipes* and *L. edodes* showed the highest ACE inhibitory activity. This was followed by *H. erinaceus*, where 90.1% of ACE activity was inhibited. About 87.2% of ACE was inhibited by the water extract of *A. bisporus*. The five *Pleurotus* spp tested exhibited 87.6 to 71.9% of ACE inhibition activity. The result obtained in the current study was comparable with the results reported in the previous study. According to the report by Kim and co-workers, 50% of the ACE activity was inhibited by *F. velutipes* at a concentration of 7.4 mg/ml while the IC<sub>50</sub> values for *L. edodes* were in the range of 18.4 to 39.3 mg/ml [19]. Meanwhile, Lee and co-workers reported 38.7% and 27.3% of ACE activity were inhibited by water extract from *P. sajor-caju* and *P. ostreatus*, respectively. The ACE inhibitory activities of *F. velutipes* were in the range of 13.7% to 32.9% while *A. bisporus* showed 27.3% of inhibition [20]. Thus, all the nine mushroom species tested in the current study are potential ACE inhibitor. Proteins were precipitated from the water extracts of the nine mushroom species for further study.

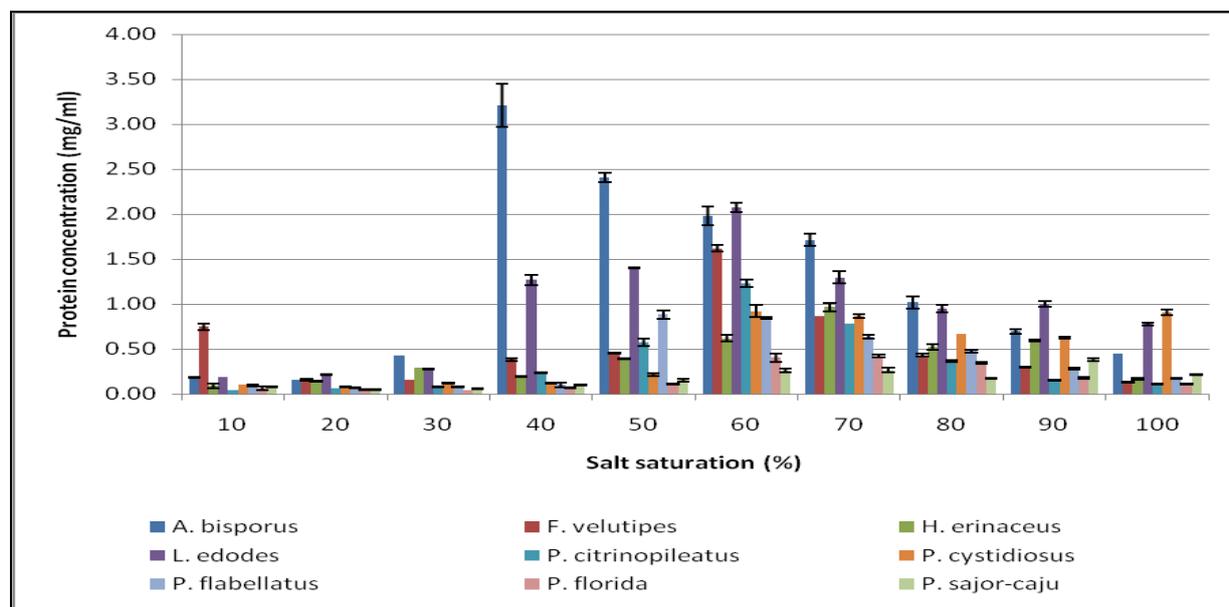
**Table 1:** Percentages of ACE inhibitory activity of water extracts from various edible mushrooms

Scientific name	ACE inhibitory activity (%)
<i>A. bisporus</i>	87.2 ± 0.8 <sup>b</sup>
<i>F. velutipes</i>	95.6 ± 0.3 <sup>g</sup>
<i>H. erinaceus</i>	90.1 ± 0.6 <sup>f</sup>
<i>L. edodes</i>	95.2 ± 0.3 <sup>g</sup>
<i>P. citrinopileatus</i>	71.9 ± 1.0 <sup>d</sup>
<i>P. cystidiosus</i>	81.3 ± 0.8 <sup>a</sup>
<i>P. flabellatus</i>	75.4 ± 1.3 <sup>c</sup>
<i>P. florida</i>	87.6 ± 1.4 <sup>b</sup>
<i>P. sajor-caju</i>	85.2 ± 0.6 <sup>e</sup>
Captopril	20.2 ± 3.2 <sup>h</sup>

Mushroom extracts were tested at 10 mg/ml. Captopril was tested at 10 ×10<sup>-8</sup> mg/ml. Each inhibitory activity is expressed as mean ± standard deviation (n=3). Means with different alphabet letters within a column denotes the ACE inhibitory activity of the samples are significantly different (P<0.05).

**Comparison of the Protein Concentrations among the Edible Mushrooms.** Protein content in mushrooms has been ranked below animal meats but well above most other foods, such as vegetables, fruits and milk [10]. In the current study, proteins were precipitated from the crude water extracts using different salt saturation ranging from 10 to 100%. More hydrophobic proteins will precipitate out first at lower salt saturations. The concentrations of the precipitated protein were estimated using BCA protein assay kit. Referring to the results shown in Fig. 1, the graph obtained showed a binomial distribution, where the amount of protein precipitated increased as the percentage of the salt saturation increased. After it reached a maximum

percentage of salt saturation, the amount of protein collected were decreased until it reached 100% salt saturation. Most of the mushroom species in the current study had the highest concentration of protein precipitated in between 50 to 70% salt saturation, except for *A. bisporus* (40% salt saturation) and *P. sajor-caju* (80% salt saturation). Thus, it can be assumed that most of the proteins contained in the mushroom fruiting bodies tested in the current study have an intermediate hydrophobicity.



**Figure 1:** Protein concentration (mg/ml) of the nine selected species of edible mushrooms at ten ammonium sulfate salt saturations.

### Comparison of the ACE Inhibitory Activity of the Partially Purified ACE Inhibitor.

Proteins of the mushroom species precipitated at different salt saturation were separated by SDS PAGE according to their molecular weight. Some of the proteins precipitated at the neighboring salt saturation showed similar bands (Data not shown). Thus, proteins precipitated at different salt saturation that showed similar protein bands were pooled together. *Hericium erinaceus* and *P. citrinopileatus* have been pooled into four groups while *A. bisporus*, *P. flabellatus*, *P. florida* and *P. sajor-caju* were divided into five groups. *Flammulina velutipes*, *L. edodes* and *P. cystidiosus* were pooled into six groups.

The ACE inhibitory activity of the protein fractions were tested at 10 µg/ml protein. Even though all the mushroom water extracts showed good antihypertensive activity (Table 1), however not all of the protein fractions showed good activity (Table 2). For example, water extract of *H. erinaceus* managed to inhibit the ACE activity at 90.1%, but protein fractions of *H. erinaceus* showed less than 7% ACE inhibitory activity. Although the purified protein has low ACE inhibitory activity, the mushrooms may still be a good ACE inhibitor due to other compounds. According to a previous study, D-mannitol in *P. cornucopiae* has been reported to exhibit a blood pressure lowering activity by inhibiting the angiotensin I-converting enzyme activity [25]. Triterpenoids in *G. lucidum* was believed to have blood pressure lowering effect [12].

Referring to Table 2, about 50% of the mushroom species tested have higher ACE inhibitory activity exhibited by hydrophobic proteins from fraction 1 compared to the other

protein fractions for the same mushroom species. This is supported by the previous report indicating that high ACE inhibitory activity is due to more hydrophobic peptides [26, 27].

Among the protein fractions of the various mushrooms, *P. cystidiosus* showed the highest antihypertensive activity where 96.2% of ACE was inhibited by fraction 1. This was followed by fraction 5 where 86.7% of ACE was inhibited. Thus, the two protein fractions have been selected for further purification of ACE inhibitory peptide.

**Table 2:** Percentages of the ACE inhibitory activity of the protein fractions from the nine selected species of edible mushrooms.

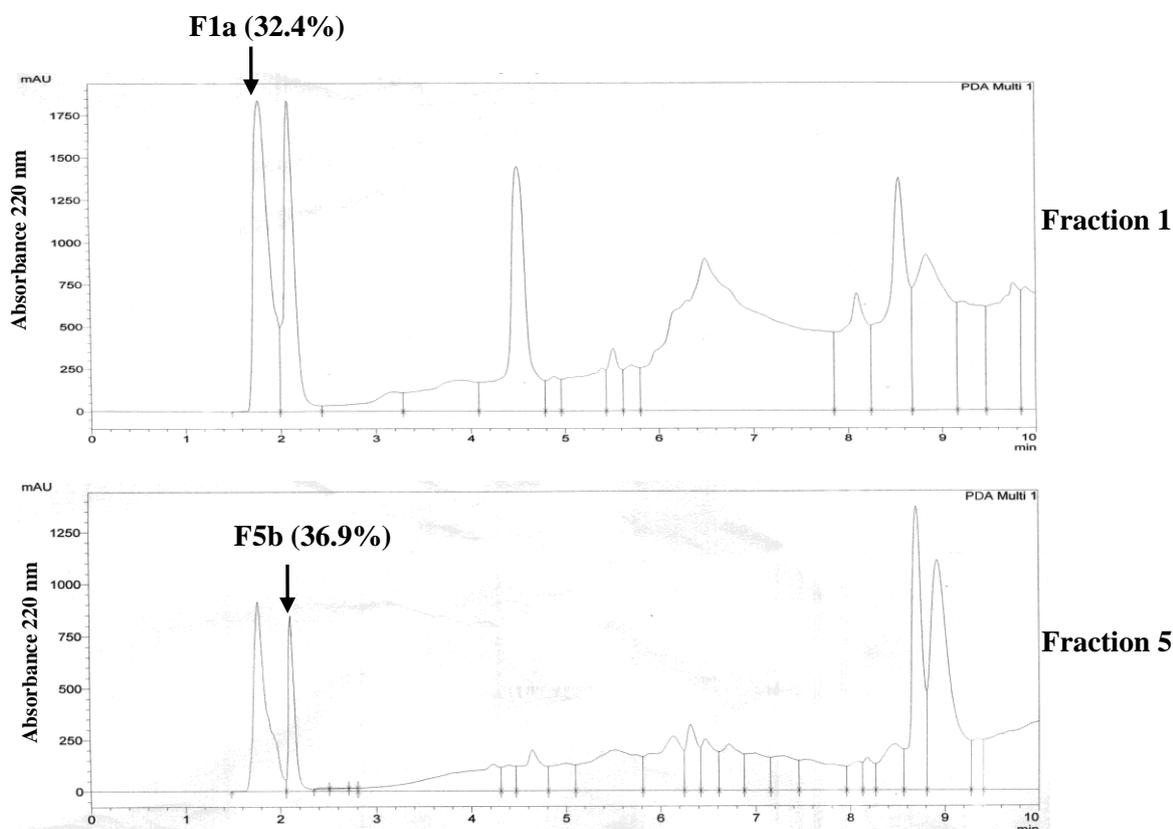
Mushroom species	Protein fractions					
	1	2	3	4	5	6
<i>A. bisporus</i>	78.4 ± 4.4	8.3 ± 3.2	71.4 ± 0.9	39.3 ± 1.3	8.1 ± 2.8	-
<i>F. velutipes</i>	18.3 ± 3.5	15.8 ± 0.8	12.6 ± 2.2	13.7 ± 3.2	16.4 ± 3.7	19.8 ± 0.5
<i>H. erinaceus</i>	5.4 ± 3.4	5.9 ± 2.9	6.3 ± 1.8	6.9 ± 4.6	-	-
<i>L. edodes</i>	11.7 ± 2.1	16.6 ± 6.3	24.9 ± 2.8	5.7 ± 2.9	3.3 ± 1.9	4.6 ± 2.6
<i>P. citrinopileatus</i>	41.1 ± 4.4	20.9 ± 1.6	15.2 ± 4.9	20.2 ± 1.5	-	-
<i>P. cystidiosus</i>	96.2 ± 0.6	47.5 ± 2.7	31.6 ± 6.1	31.5 ± 2.3	86.7 ± 2.8	48.3 ± 2.6
<i>P. flabellatus</i>	28.4 ± 3.1	36.5 ± 4.1	21.8 ± 3.9	19.9 ± 3.0	22.0 ± 3.4	-
<i>P. florida</i>	25.5 ± 2.3	24.2 ± 8.5	17.0 ± 3.1	22.9 ± 4.7	4.5 ± 4.2	-
<i>P. sajor-caju</i>	41.2 ± 7.3	30.6 ± 0.8	35.9 ± 2.2	32.2 ± 4.2	5.5 ± 1.7	-

Protein fractions were tested at 10 µg/ml protein. Each inhibitory activity is expressed as mean ± standard deviation (n=3).

**Purification of ACE Inhibitor from *P. cystidiosus* by RPHPLC.** Fraction 1 and 5 of *P. cystidiosus* which showed the highest ACE inhibitory activity were selected for further purification by RPHPLC. The RPHPLC chromatogram for the two protein fractions are shown in Fig.2. The ACE inhibitory activity of the collected RPHPLC fractions was tested at a concentration of 1 µg/ml protein. The result obtained showed lower antihypertensive activity than expected. This indicates that there may be a synergistic action among the peptides when present together in the whole protein fractions. This synergistic effect has also been proposed for active ACE inhibitory peptides isolated from cheese and tilapia [28, 29]. RPHPLC peaks collected from fraction 1 inhibited the ACE activity in the range of 3.3 to 32.4%. The highest activity was exhibited by fraction F1a, which was eluted at 1.7 min. RPHPLC peaks collected from fraction 5 inhibited the ACE activity in the range of 17.4 to 36.9%. The highest activity was exhibited by fraction F5b, which was eluted at 2.1 min. Thus, the two protein sub-fractions have been selected for SELDI profiling.

**SELDI Profiling of the Partially Purified ACE Inhibitor from *P. cystidiosus*.** In the last few years, proteomics profiling experiments were performed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled to mass spectrometry (MS). Previous study has reported on the protein profiling of mushroom using the 2D-PAGE MS technique [30]. However, this technique is laborious and limits the isolation of low-abundant proteins. Besides, it requires large

amounts of samples [31]. Thus, to overcome the limitations of 2D-PAGE MS, SELDI-TOF-MS has been used for the protein profiling analysis in the current study.

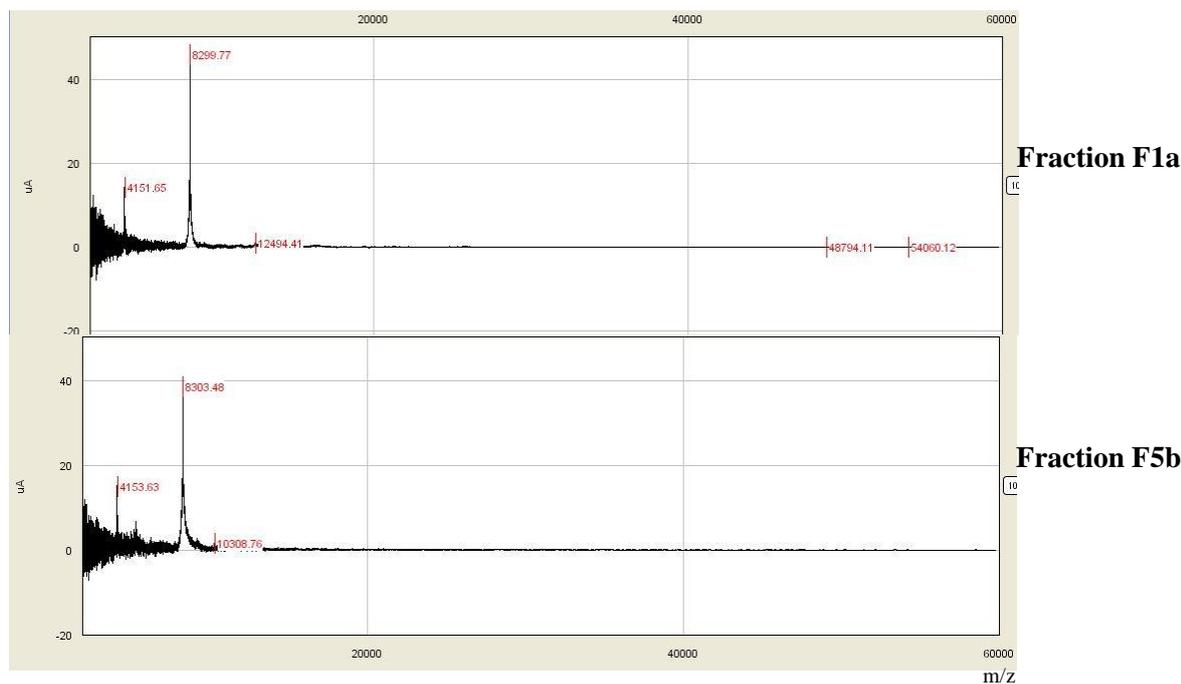


**Figure 2:** RPHPLC chromatogram of fraction 1 and fraction 5 of *P. cystidiosus*. Sub-fraction with the highest ACE inhibitory activity and the percentages of inhibition are presented.

Referring to Figure 3, there were five proteins observed in fraction F1a with the m/z of 4151.65, 8299.77, 12494.41, 48794.11 and 54060.12. Three proteins were observed in fraction F5b with m/z of 4153.63, 8303.48 and 10308.76. In both fractions, the peak with m/z of 8300 has the highest intensity followed by m/z of 4100. Hence, the ACE inhibitory activity of the two fractions might be due to these low molecular weight proteins. Previous studies had reported on the higher ACE inhibitory activity exhibited by lower molecular weight peptides [32, 33]. Protein with large molecular size was reported to have disadvantage of metabolic instability. Thus, it is not suitable to be used as cardiovascular drug [34]. Peptides with two or three amino acid residue lengths could be absorbed directly from the digestive tract into the blood circulatory system and be able to reach the action sites to exert physiological functions [35]. Hence, the low molecular weight protein from *P. cystidiosus* could be a good source of ACE inhibitor.

## CONCLUSION

Edible mushrooms can be a good source of bioactive compounds with ACE inhibitory activity. Among the nine mushroom species tested in the current study, protein fractions from *P. cystidiosus* had the highest ACE inhibitory activity which may be due to low molecular weight protein. Although the ACE inhibition effect is lower compared to captopril, the inhibitor from *P. cystidiosus* is a peptide derived from a food source that can be eaten daily. Further studies have to be carried out to identify the peptide sequence of the ACE inhibitor.



**Figure 3:** SELDI-TOF-MS spectra for protein sub-fractions F1a and F5b of *P. cystidiosus* generated using H50 ProteinChip array.

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